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Dilatory is a *Drosophila* protein related to AZI1 (CEP131) that is located at the ciliary base and required for cilium formation

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Summary

A significant number of ciliary disease genes have been found to encode proteins that localise to the basal body. By contrast, a large number of basal-body-associated proteins remain to be characterised. Here, we report the identification of a new basal body protein that is required for ciliogenesis in *Drosophila*. Dilatory (DILA) is a predicted coiled-coil protein homologous to vertebrate AZI1 (also known as CEP131). Mutations in *dila* specifically exhibit defects in ciliated cells (sensory neurons and sperm). Several features of the neuronal phenotype suggest a defect in intraflagellar transport. In sensory neuron cilia, DILA protein localises to the ciliary base, including the basal body and putative transition zone, and it interacts genetically with the ciliary coiled-coil protein, Uncoordinated. These data implicate DILA in regulating intraflagellar transport at the base of sensory cilia.

Key words: Cilia, *Drosophila*, Basal body, Sensory neuron, Spermatozoa

Introduction

Cilia are highly structured eukaryotic organelles that perform a variety of motile and sensory functions in development and physiology (Satir and Christensen, 2007; Silverman and Leroux, 2009). Studies of model organisms, such as *Chlamydomonas reinhardtii* and *Caenorhabditis elegans*, have greatly advanced our knowledge of ciliogenesis, particularly of intraflagellar transport (IFT) – the specialised trafficking process by which ciliary proteins and vesicles move through the cilium during cilium assembly at the surface of the cell (Dawe et al., 2006; Marshall, 2008; Rosenbaum and Witman, 2002; Silverman and Leroux, 2009; Stephan et al., 2007). Such studies have established that anterograde transport of ciliary cargoes involves kinesin-2 and the IFT-B subcomplex of proteins, whereas retrograde transport requires cytoplasmic dynein-2 and IFT-A proteins (Rosenbaum and Witman, 2002). IFT is required not only to construct the cilium but also for establishing and maintaining the correct localisation of receptor proteins and ion channels.

In *Drosophila*, cilia are confined to just two cell types – sensory neurons and spermatozoa – which facilitates in vivo analysis of ciliogenesis (Laurençon et al., 2007; Lee et al., 2008). Type I sensory neurons have sensory processes that terminate in a modified cilium, which is the site of sensory reception and transduction (Field and Matheson, 1998; Keil, 1997). The cilium of chordotonal (Ch) mechanosensory neurons has a typical 9+0 axonemal pattern of microtubule doublets. A swelling known as the ciliary dilation divides the Ch cilium into proximal and distal segments, which each have distinct compositions of ion channels (Gong et al., 2004;

Kim et al., 2003; Lee et al., 2010). Moreover, in the proximal cilium, each microtubule doublet is furnished with dynein arms, which are linked to mechanically induced motility (Kavlie et al., 2010). The IFT machinery is well conserved in *Drosophila* (Avidor-Reiss et al., 2004), and several components have been characterised by virtue of the sensory deficits that result from their mutation (Han et al., 2003; Lee et al., 2008). Such analyses have yielded insights that were not apparent from protist studies, such as the involvement of an IFT-A protein in maintaining the functionally distinct segments of the Ch cilium (Lee et al., 2008).

Apart from Type I sensory neurons, the only other cells in flies that bear cilia are the sperm – the flagellum being a modified cilium. *Drosophila* spermatids arise from spermatocytes that undergo meiosis and differentiation (including flagellum assembly) within a syncytial cyst before the spermatids become bounded ('individualised') by separate membranes (Fuller, 1993). This radically different method of ciliogenesis does not involve IFT (Han et al., 2003; Lee et al., 2008; Sarpal et al., 2003). Thus *Drosophila* is a useful model for dissecting different modes of ciliogenesis.

The basal body, a membrane-tethered derivative of the centriole, is the site of assembly and remodelling of the cilium (Dawe et al., 2006). As well as anchoring the cilium, the basal body is thought to provide a selective gateway regulating the entry of ciliary proteins and vesicles by IFT, and basal body defects therefore lead to truncated cilia (Dawe et al., 2006; Marshall, 2008; Rosenbaum and Witman, 2002; Silverman and Leroux, 2009; Stephan et al., 2007). Little is yet known about how basal body structure and molecular composition relate to this function, although some recent progress has been made in protists (Craigie et al., 2010; Graser et al., 2007; Lechtreck et al., 1999). Moreover, the basal body is a highly complex entity, and proteomic and comparative genomic analyses have identified large numbers of centrosome- or basal-

body-associated proteins (Andersen et al., 2003; Gherman et al., 2006; Keller et al., 2005; Kilburn et al., 2007; Laurençon et al., 2007; Li et al., 2004), but little is known about how they contribute to the structure and function of this region of the cilium. The part of the cilium just distal to the basal body also appears highly specialised, with axoneme-membrane linkers and membrane elaborations. This region has been termed the transition zone, and, like the basal body, it has been proposed to play a role in IFT regulation (Craig et al., 2010; Omran, 2010; Seeley and Nachury, 2010).

So far, only one basal-body-specific protein has been studied in *Drosophila* (Baker et al., 2004). This protein, Uncoordinated (UNC), is required for ciliogenesis but is not conserved, so its general significance is unclear. Here, we report the characterisation of a new gene that encodes an evolutionarily conserved basal-body-associated protein. This protein, Dilatory (DILA), is a coiled-coil protein that is homologous to vertebrate AZI1 (also known as CEP131). Tagged DILA localises to the base of sensory neuron cilia. Mutations in *dila* result in flies with ciliary defects in both sensory neurons and sperm. Our analysis of this phenotype suggests that DILA participates in the regulation of transport at the base of the cilium in collaboration with UNC.

Results

dila encodes a coiled-coil protein and is expressed in all ciliated cells

In a microarray screen for genes expressed during the development of Ch neurons, we identified *CG1625*, which we named *dilatory* (*dila*) (Cachero et al., 2011) (Fig. 1A). *dila* has two predicted transcripts, *CG1625-RA* (3381 bp) and *CG1625-RB* (3480 bp) (Fig. 1A). RT-PCR shows that both transcripts exist in fly embryos (L.M. and A.P.J., unpublished work). The *RA* cDNA sequence was identical to that predicted by FlyBase annotation, except that 21 bp of the predicted first intron were found to be part of the second exon (as is predicted already for *RB*). The *RB* cDNA seemed to differ from that defined by FlyBase annotation by including the entire predicted first intron of that transcript. *CG1625-RA* encodes a predicted protein of 1134 amino acids. The predicted protein has a C-terminal region that is highly conserved among *Drosophila*

species and contains five coiled-coil domains (residues 765–1131). The protein shares 18.6% identity (30.4% similarity) with the mouse coiled-coil protein, AZI1 (Aoto et al., 1996) [also known as CEP131 (Andersen et al., 2003)]. Within the coiled-coil domains, this rises to 27.5% identity (42.2% similarity), suggesting that DILA is the AZI1 orthologue.

During embryogenesis, *dila* mRNA was expressed in developing and differentiating ciliated sensory neurons of both the Ch and external sensory (ES) lineages (Fig. 1B–D). Expression began in the Ch precursor cells before their division and persisted until at least the end of embryogenesis in the differentiated Ch neurons. Expression in ES neurons was generally more transient. To determine whether *dila* is associated with ciliogenesis in general, we examined expression in the adult testis. Here, *dila* mRNA was expressed in the cell bodies of elongating spermatids (Fig. 1E). In situ hybridisation did not rule out the possibility that *dila* was also expressed at a low level in other cells, but reporter gene analysis using the entire intergenic region upstream of *dila* supported the conclusion that *dila* is expressed exclusively in the Ch and ES lineages (Cachero et al., 2011). Moreover, by microarray analysis, *dila* was the highest ranked differentially expressed gene in mid-staged embryonic Ch cells (Cachero et al., 2011). Thus, although we cannot completely exclude low-level expression in other cells, the evidence suggests that *dila* is expressed exclusively in ciliated cell types.

dila mutant flies are uncoordinated

To generate *dila* mutations, we mobilised a nearby P-element (P{GT1}CG30001^{BG02674}) to create imprecise excisions extending in the direction of the *dila* gene. Three 3' deletions were generated, of 0.9, 1.4 and 2.0 kb, respectively, in which all or most of the DNA encoding the C-terminal coiled-coil domain was deleted (Fig. 1A). Homozygous embryos showed reduction in mRNA levels that correlated with deletion size, suggesting an increasing instability of the truncated transcripts (supplementary material Fig. S1). Most of the following analysis is based on the largest deletion, *dila*⁸¹, which is likely to be a strong hypomorph or a null.

For all three *dila* deletions, homozygous individuals reached the adult stage, and these mutant flies showed uncoordinated behaviour.

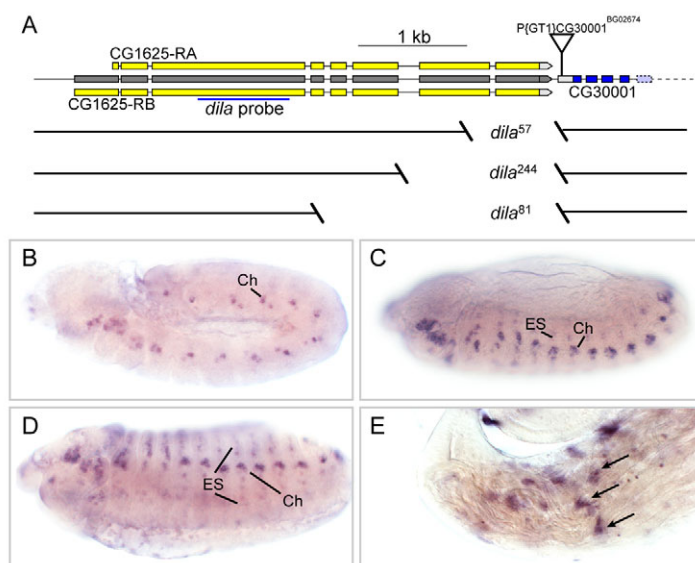


Fig. 1. The *dila* gene and its expression. (A) Molecular map of the *dila* (*CG1625*) region from FlyBase and our RT-PCR studies. Indicated are the transcripts, the probe used for in situ hybridisation (blue line) and the P-element insertion site within the adjacent *CG30001* gene, from which three excision deletion alleles were generated. Each deletion removes part of the 3' end of the *dila* gene, including the coiled-coil motifs. (B–E) mRNA in situ hybridisation with *dila* probe. (B) At stage 11 *dila* is expressed first in Ch neural precursors. (C) Expression continues in both Ch and ES sensory neuron lineages at stage 14. (D) At stage 15 to 16 expression persists in the Ch and ES neurons. (E) In the distal adult testis *dila* mRNA is located in the heads of each individual cyst, where the nuclei of spermatids cluster.

Mutant flies had a dramatically reduced climbing ability in bang tests compared with wild-type controls (Fig. 2A). In addition, flies homozygous for the largest deletion, *dila*⁸¹, were incapable of standing or righting themselves (supplementary material Movie 1); they died very soon after eclosion. Coordinated locomotion depends on sensory feedback (Caldwell et al., 2003). Given the expression pattern of *dila*, these phenotypes therefore suggest a defect in sensory neuron structure or function. The uncoordination of *dila* mutant flies was more severe than that observed for *atonal* mutants, which lack Ch organs (Fig. 2A). This suggests that all types of ciliated neuron (both Ch and ES) are defective in *dila* mutants, as is observed for ciliogenesis genes such as *nompB* and *rempA* (Han et al., 2003; Lee et al., 2008).

The P-element used to generate *dila* deletions was inserted at the 5' end of an adjacent gene, *CG30001*, the function of which is unknown (Fig. 1A). To establish whether *CG1625* was responsible for the observed phenotypes rather than *CG30001*, we carried out a rescue experiment. A fragment containing the *CG1625-RA* open reading frame (ORF) was inserted into a UAS vector in frame with

a multimer FLAG tag, and transformant lines generated. *dila*⁸¹ mutant flies containing UAS-*dila*-FLAG driven by either *elavGal4* or *scaGal4* showed a strong rescue of the locomotion phenotype (supplementary material Movie 1).

dila mutants have defective sensory cilia

In *dila*⁸¹ mutants, Ch neurons are grossly normal in most respects (L.M. and A.P.J., unpublished) but show defects in their ciliary sensory processes. To study the ciliary morphology of Ch neurons, we examined the ultrastructure of Johnston's organ (JO) – a large array of Ch organs in the adult antenna. Each scolopale of JO houses the ciliary sensory processes of two or three Ch neurons (Fig. 2B). Cilia of *dila*⁸¹ Ch neurons showed a range of ultrastructural defects. Some cilia were shortened, thereby appearing missing in cross-section (Fig. 2C,D). Other cilia were present but usually had a disorganised axonemal structure, often with missing microtubule doublets (Fig. 2G,H), or were occasionally grossly abnormal in structure or orientation (Fig. 2O). In addition, the doublets mostly lacked their associated dynein arms (Fig. 2E,F),

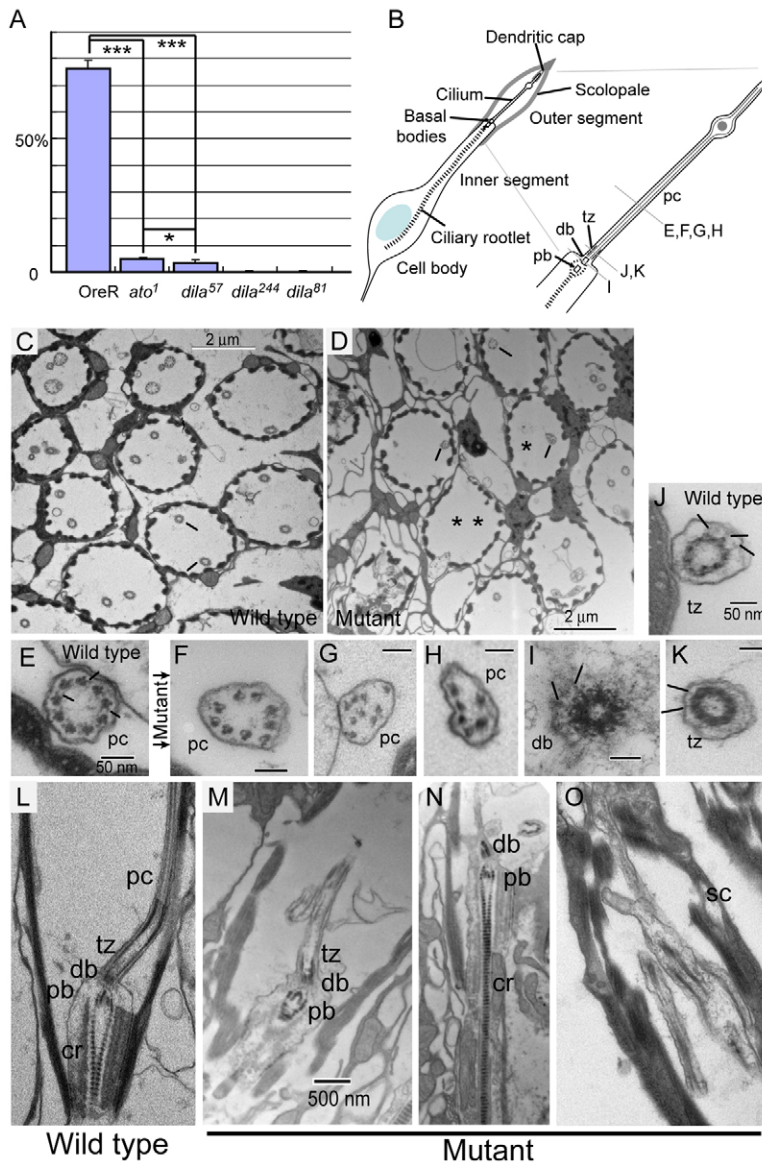


Fig. 2. Ciliary defects in *dila* mutant flies. (A) The number of flies climbing above a threshold in a bang test. Very few *dila* mutant flies show climbing ability compared with wild-type flies (OreR). *ato* mutant flies, which lack Ch organs, also fail this test. *** $P < 0.001$; * $0.01 < P < 0.05$ (Student's *t*-test).

(B) Schematic of a Ch scolopidium, showing the locations of transverse sections. Shown are ciliary rootlet (cr), proximal basal body (pb), distal basal body (db), transition zone (tz) and proximal cilium (pc). (C–K) Electron micrographs of transverse sections through antennal Ch scolopidia. (C) Wild-type scolopales (sc) contain cilia from two or three neurons. (D) Cilia in *dila*⁸¹ Ch organs are variably disrupted. Some cilia are absent in cross-section, indicating truncation (asterisk), whereas others have an abnormal axoneme structure. (E) Transverse section through a wild-type proximal cilium (pc) showing the 9+0 axoneme with dynein arms (indicated). (F) These are missing in the mutant. (G,H) *dila* mutant cilia showing disorganisation or reduction of microtubule doublets. (I) A *dila* mutant distal basal body (db) appears normal (transitional fibres indicated). (J) Wild-type putative transition zone (tz) showing electron-dense axoneme ring (possible fibrous appendages indicated). (K) Mutant transition zone. (L–O) Longitudinal sections through antennal Ch scolopidia. (L) Wild-type cilium base. (M,N) *dila* mutant showing grossly intact basal structures. (O) Grossly malformed cilia in a *dila* mutant.

which are thought to be required for the ciliary beat that occurs in response to mechanical stimulation and acts as a signal amplification mechanism (Göpfert and Robert, 2003; Kavlie et al., 2010). The disorganised axoneme patterns suggest that other axoneme-associated components are also missing, given that loss of dynein arms alone does not alter axoneme organisation (Kavlie et al., 2010). In contrast to the ciliary compartment, basal sections showed no obvious defects in the ciliary rootlet or basal bodies and their associated fibrous structures (Fig. 2I–M). Beyond the basal bodies is a region in which the axoneme forms an electron-dense ring in transverse sections (Fig. 2J,L). In studies of Ch neurons in other insects, this region (termed the ‘ciliary base’) was found to have delicate ‘champagne-glass-shaped extensions’ connecting the axoneme to the membrane (Young, 1973). We suggest that at least part of this region corresponds to the transition zone of other cilia (Omran, 2010; Seeley and Nachury, 2010), and here we refer to it as the putative transition zone. This region showed no obvious defects in *dila* mutant cilia, although the fibrous structures were difficult to detect in our hands (Fig. 2J,K). In summary, in *dila*⁸¹ mutants, ciliary defects appear confined to the cilium beyond these basal structures.

In *dila*⁸¹ embryos and larvae, Ch neuronal cilia were also missing or shortened as judged by anti-horse radish peroxidase (HRP) antibody staining. The anti-HRP antibody recognizes sugar residues on multiple glycoproteins that are transported into the Ch neuron cilium and secreted into the scolopale lumen to form two bands (Jan et al., 1985) (Fig. 3A,B,F). In addition to highlighting the shortened cilia, HRP immunoreactivity was strikingly accumulated at the base of the cilia in embryos (Fig. 3C) and larvae (Fig. 3D,E). Another scolopale lumen protein, Eyes shut (EYS) (Husain et al., 2006), showed a similarly abnormal basal accumulation (Fig. 3D,E). The deposit of EYS at the base of the cilia was also seen in the IFT-associated dynein mutant, *beethoven* (*biv*) (Lee et al., 2008). Together, this and the defects observed by electron microscopy suggest a disruption of transport into the cilium.

Spermiogenesis is defective in *dila* males

To explore whether *dila* is involved in ciliogenesis generally, we examined sperm production (Han et al., 2003). We tested the

fertility of flies in which peripheral nervous system (PNS) defects had been substantially rescued by UAS-*dila*-FLAG expression (see above). Male PNS-rescued flies showed a very low level of fertility, suggesting a primary defect in spermatogenesis (Fig. 4A). By contrast, PNS-rescued females show a normal level of fertility (L.M. and A.P.J., unpublished).

*dila*⁸¹ mutant testes are of normal size and shape but few, if any, mature motile sperm were evident in the 2-day-old adult testis compared with wild type (data not shown). Phase-contrast microscopy of *dila*⁸¹ testis squashes ($n=11$) showed no defects in meiotic cysts and a very low level of defects in onion-stage spermatocytes (seven examples had one to four abnormal cells in a single 64-cell cyst; data not shown). This suggests that defects occur after mitosis and meiosis and during sperm differentiation (spermiogenesis). During the elongating stage (when flagella are formed), sperm nuclei showed substantial disorganisation in the *dila*⁸¹ testis. In wild-type syncytial cysts, nuclei tightly associate with each other to form a well-organised cluster (Fig. 4B,C). This organisation was strongly disrupted in the *dila*⁸¹ testis, with some nuclei misorientated within the nuclear clusters and others dispersed along the flagellar bundles (Fig. 4D,E). In addition, substantial disorganisation of the flagellar bundles was observed (Fig. 4H).

Several basal body markers showed impaired localisation in *dila* mutant sperm. GFP-pericentrin/AKAP450 centrosomal targeting domain (PACT) is a marker of the rod-shaped centriole (Martinez-Campos et al., 2004), whereas γ -tubulin labels the collar-shaped centriolar adjunct surrounding the centriole (Blachon et al., 2008; Wilson et al., 1997). In wild-type sperm these locate to the apical tip of the sperm nuclei (Fig. 4C,F). In *dila* mutant sperm, both markers were often abnormal in location (Fig. 4D,G), with γ -tubulin often missing from the scattered nuclei (Fig. 4E). Similar defects were observed for the coiled-coil protein YURI, which is proposed to be required for correct attachment of the basal body to the nuclear membrane during differentiation (Texada et al., 2008).

Electron microscopy confirmed that sperm were often chaotically organised within their cysts in *dila* mutants (Fig. 4I). There were also individualisation defects, with many sperm tails sharing syncytial cytoplasm or lacking surrounding cytoplasmic membrane (Fig. 4I,J). Possibly as a consequence, many flagella were found

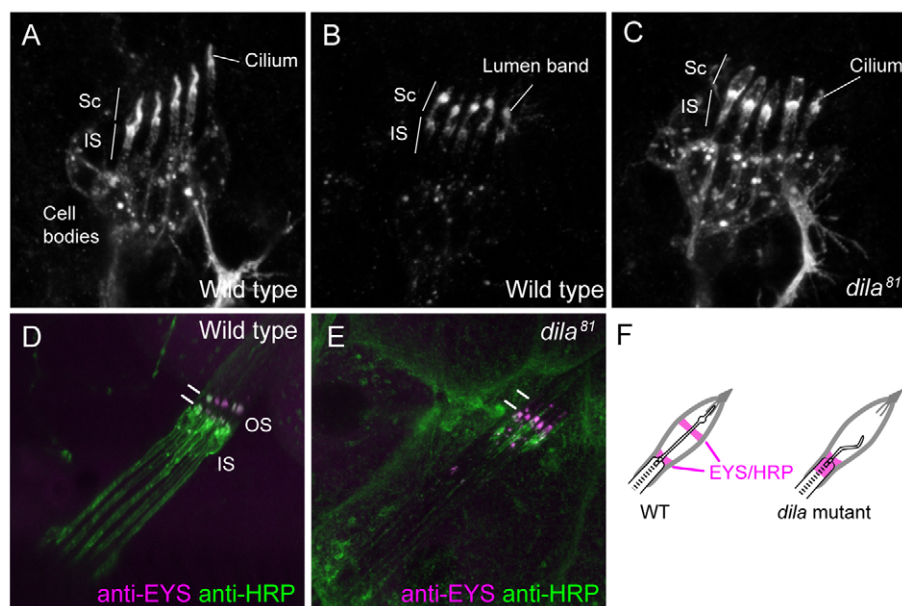


Fig. 3. Protein localisation in *dila* mutant cilia.

(A,B) Wild-type embryonic Ch neurons stained with anti-HRP antibody, showing expression in the cilia (A) and subsequently in the scolopale (sc) lumen band (B). (C) In the *dila*⁸¹ mutant, anti-HRP antibody reactivity largely remains at the base of truncated cilia. (D,E) Expression of HRP and EYS in larval Ch neurons. (D) Wild-type. (E) *dila* mutant. (F) Schematic summary of EYS and HRP staining. IS, inner segment; OS, outer segment.

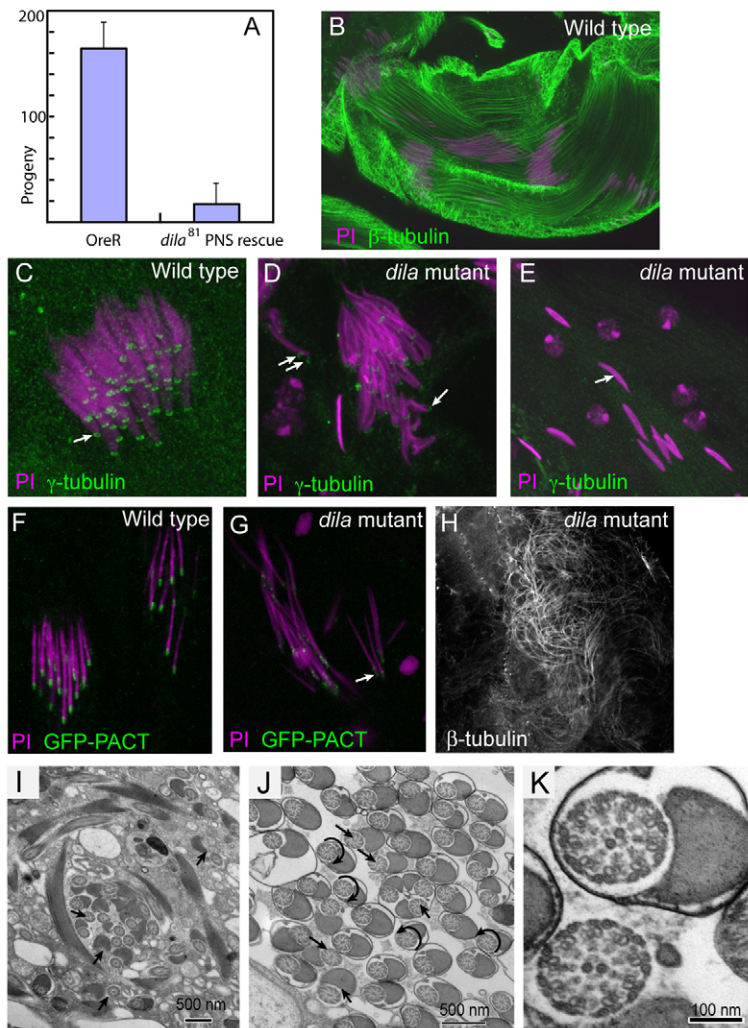


Fig. 4. Defects in sperm formation. (A) Progeny produced by single wild-type (OreR) and PNS-rescued *dila* mutant males when crossed to wild-type females. (B) Wild-type testis with organised and orientated sperm bundles. Nuclei are labelled with propidium iodide (PI, magenta) and flagella with β -tubulin (green). (C) A wild-type cyst of elongating spermatids, showing nuclei (PI, magenta) and their apical centriolar adjuncts are labelled with γ -tubulin (green). (D) In *dila*⁸¹ cysts of elongating spermatids, the nuclei are misorientated and disorganised with many bundles and centriolar adjuncts misplaced (arrows). (E) In other cases the cyst is disrupted with scattered nuclei that often lack γ -tubulin staining (arrow). (F) In wild-type cysts GFP-PACT labels the rod-shaped centriole. (G) In *dila* mutants GFP-PACT is irregularly localised. (H) β -Tubulin staining of flagella shows chaotic organisation of mutant sperm. (I–K) Transverse sections through elongating spermatids from *dila* mutant testis. (I) In many cases spermatids fail to individualise and are chaotically organised, and many show separation of the axonemes from their mitochondria (arrows). (J) A relatively well-formed cyst shows some individualisation failures (lack surrounding membranes; straight arrows). Axonemal polarity differences (curved arrows) are consistent with differing orientations of sperm within the cyst or looping of sperm flagella. (K) Higher magnification shows well-formed axonemal structures of both individualised (upper) and non-individualised (lower) sperm.

separated from their mitochondrial derivatives (Fig. 4J). Mixed axoneme polarities were observed, consistent with flagella lying in both orientations within the same elongating spermatid cyst (Fig. 4J). However, despite these defects, flagellar axoneme structure appeared intact – an observation that is in striking contrast to Ch neuronal cilia (Fig. 4J,K).

DILA is localised to the base of the ciliary processes of Ch neurons

To indicate the site of *dila* function in sensory neurons, we investigated the subcellular localisation of DILA protein. When expressed in larval Ch neurons using a *scaGal4* driver, FLAG-tagged DILA protein (DILA-FLAG) localised to a pair of dots at the base of the cilia (Fig. 5A). To explore this, we examined the location of DILA-FLAG relative to Sas-4 (Basto et al., 2006). This protein is a centriole marker in *Drosophila* embryonic cells, and *Sas-4* mutant flies have ciliogenesis defects that lead to the uncoordinated behaviour. We established that, after neuronal differentiation, Sas-4 was localised at the distal and proximal basal bodies (Fig. 5B). Co-labelling showed that DILA-FLAG localisation overlapped with Sas-4, but was often strongest between and beyond the Sas4-positive foci (Fig. 5B and data not shown). The most consistent and prominent localisation was in the cilium just beyond the distal basal body. This corresponds to the ciliary

base region described above as being putatively equivalent to the transition zone (Fig. 5B,D). Occasionally, variable protein accumulation was observed in the cilium itself (Fig. 5A) but this appeared to be dependent on the expression strength of the DILA-FLAG protein, suggesting that it is an artifact of protein overexpression. To minimise this possibility, we examined DILA-FLAG insertion lines that had a low level of expression (i.e. leaky expression) in the absence of a *Gal4* driver gene. One such line showed a barely detectable level of protein in Ch neurons. In embryos, this expression was basal-body-associated (data not shown), whereas in larvae it was specifically confined to the region distal to Sas-4, suggesting localisation at the putative transition zone (Fig. 5C).

Interestingly, *dila* mRNA expression was present in undifferentiated precursors and their daughter cells (Fig. 1B), suggesting that the protein might also be translated before cell cycle exit and differentiation. When expressed in precursor cells, the DILA-FLAG fusion protein colocalised at centrosomes with the pericentriolar material (PCM) marker CP190 (Fig. 6) (Butcher et al., 2004). The completeness of this colocalisation suggests that DILA-FLAG is associated with the PCM rather than with the centriole itself, which is usually observed as a defined dot surrounded by the PCM. Despite this centrosomal localisation, neuronal cell division is normal in *dila*⁸¹ embryos, as judged by the

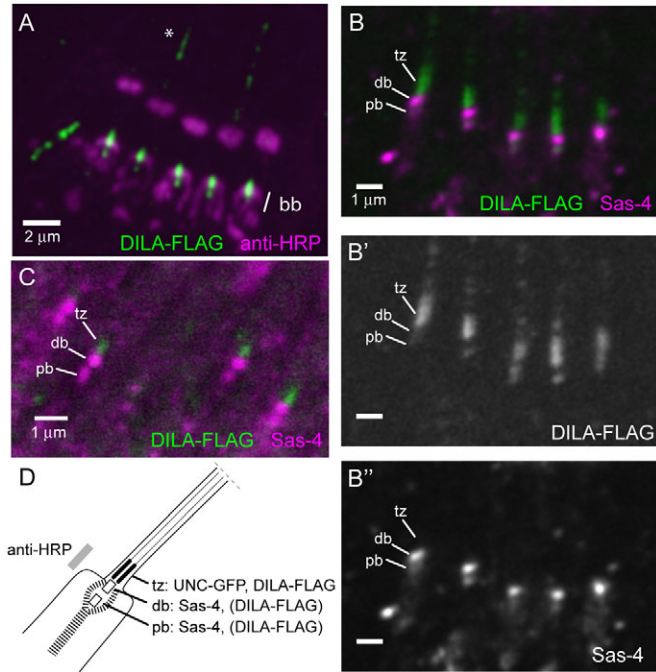


Fig. 5. DILA-FLAG resides at the base of the cilium. (A) When expressed in larval Ch neurons, DILA-FLAG is localised at the basal body (bb) region, as indicated by anti-HRP antibody staining. The DILA-FLAG staining in the distal cilia (*) varies with the expression level and is presumed to be an artifact of protein overexpression. (B–B'') Sas-4 (magenta) reveals the distal and proximal basal bodies (db, pb) at the base of each cilium. DILA-FLAG staining overlaps with Sas-4 staining and is especially prominent in the cilium beyond the distal basal body, predicted to be the putative transition zone (tz). (C) When expressed at a low level, DILA-FLAG is confined to the putative transition zone. (D) Schematic of the cilium base showing the distribution of proteins.

pattern of neurons in the late embryonic PNS (L.M. and A.P.J., unpublished). Moreover, the distribution of centrosome markers [CP190, *Drosophila* pericentrin-like protein (dPLP), TACC and γ -tubulin] is not altered in *dila*⁸¹ sensory precursor cells (Gergely et al., 2000; Martinez-Campos et al., 2004) (L.M. and A.P.J., unpublished). Thus *dila* is not required to organise centrosomes prior to differentiation.

DILA and UNC interact at the base of sensory neurons

UNC was reported as a new *Drosophila* protein that might play a role in the transformation of the centriole to the basal body (Baker et al., 2004). Like DILA, UNC is a coiled-coil protein that is required for ciliogenesis (Baker et al., 2004) and is present at sensory precursor centrosomes prior to neuronal differentiation (Cachero et al., 2011) before becoming localised to the distal and proximal basal bodies of late embryonic Ch neurons (Baker et al., 2004). However, when UNC-GFP localisation was examined in mature larval Ch neurons, we found that UNC-GFP moves distally from the basal bodies, suggesting that it also localised at the putative transition zone (Fig. 7A). These similarities between UNC and DILA led us to ask whether UNC and DILA require the function of each other for correct localisation. When expressed in *dila*⁸¹ mutant larvae, UNC-GFP was delocalised and, in fact, not visible in Ch cilia ($n=15$ larvae) (Fig. 7B,C), suggesting that either UNC-GFP protein is unstable when not localised or it is too

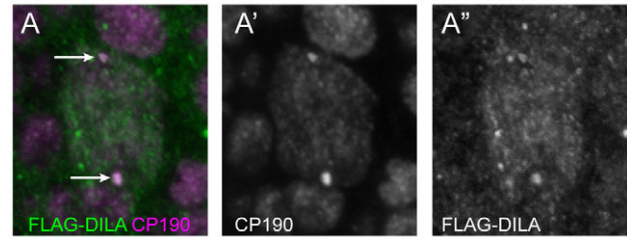


Fig. 6. DILA-FLAG is associated with centrosomes in dividing Ch precursor cells. (A–A'') A dividing Ch sense organ precursor expressing DILA-FLAG (*scaGal4*, UAS-*dila*-FLAG). Some of the protein (green) colocalises with the pericentriolar marker, CP190 (magenta).

widely dispersed to be detectable. Similarly, when DILA-FLAG was expressed in *unc*²⁴ mutant larvae, it was strongly delocalised: either it diffused along the distal tip of cilium or, otherwise, formed aggregates in the dendrites (Fig. 7D). Thus, DILA and UNC require each other for stable localisation at the base of the cilium. By contrast, mutation of *dila* or *unc* did not detectably alter the localisation of the core centriole and basal-body protein, Sas-4 (Fig. 7E,F). This suggests that DILA and UNC perform a function distinct from that of the core proteins of the basal body that are inherited from the centrosome – a conclusion consistent with the absence of overt basal body structural defects in *dila* mutant Ch neurons.

The hypothesis that DILA and UNC function together in the transition zone was explored in genetic interaction experiments using *dila*²⁴⁴, a moderate allele with a mildly uncoordinated phenotype (supplementary material Movie 1). When one copy of the *unc* gene was mutated in *dila*²⁴⁴ homozygote flies, their uncoordinated phenotype became much more severe (Fig. 7G). This suggests that these two proteins function together in a common pathway or process at the base of the cilium, and they might potentially interact directly.

Discussion

The basal body is a complex structure with functions in membrane attachment, axoneme templating and ciliary transport docking (Marshall, 2008). Several proteins are known to be recruited to the basal body during its conversion from the centrosome, and are required for basal-body-specific functions (Bettencourt-Dias and Carvalho-Santos, 2008). Our data suggest that DILA belongs to this group of proteins: DILA-FLAG is localised to the basal body and putative transition zone, and *dila* mutants show defects in cilia. Mutations of general centrosomal proteins can also be expected to cause ciliary defects, in these cases due to absent or defective centrosomes or basal bodies (Basto et al., 2006; Blachon et al., 2008; Martinez-Campos et al., 2004). The evidence suggests that *dila* is not generally required for centrosome duplication or maturation: expression outside of sensory neurons and sperm is very low or absent, *dila* mutants do not show defects in PCM assembly or male meiosis, and basal body structure appears normal. Nevertheless, *dila* mRNA is expressed before ciliogenesis in dividing Ch neural precursors and DILA-FLAG can localise to centrosomes in these cells. It is possible that acquisition of DILA is an early step in converting the centrosome into basal body.

What is the role of DILA at the base of the cilium? In Ch neurons, several aspects of the *dila* mutant phenotype suggest an insufficiency in transport of components into the growing cilium.

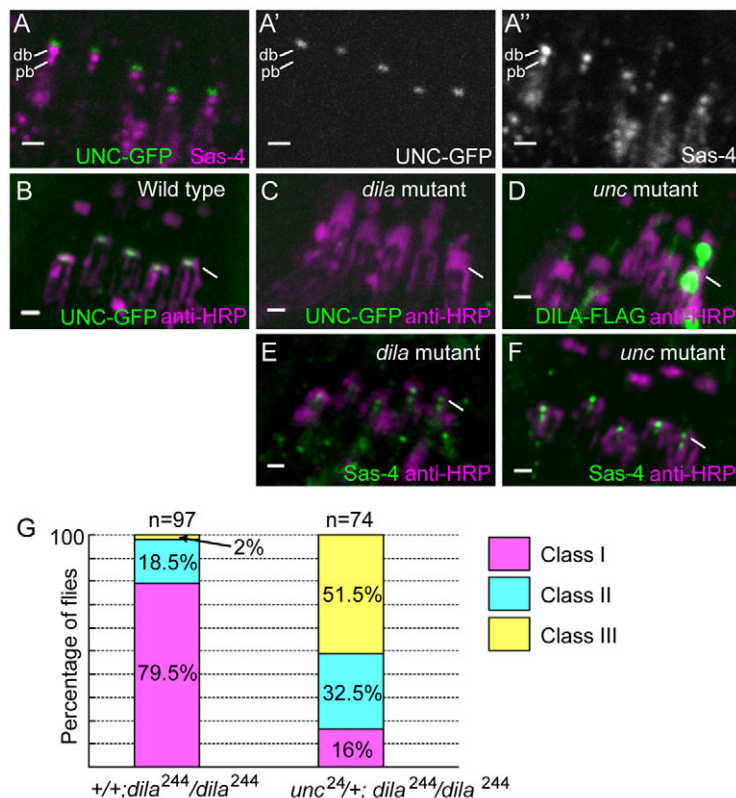


Fig. 7. Co-dependent localisation of DILA-FLAG and UNC-GFP. (A–F) Larval Ch neurons. (A) The UNC-GFP fusion protein (green) is detected in the region just above the distal basal body, as marked by Sas-4 expression (magenta). This is predicted to be the transition zone. (B) UNC-GFP in wild-type flies, co-stained with anti-HRP (magenta). (C) In *dila*⁸¹ mutants, UNC-GFP is absent. (D) In *unc*²⁴ mutants, DILA-FLAG staining is weak and diffuse, with little detectable in the basal body region (compare with Fig. 5B), except for some abnormal aggregations in one dendrite. By contrast, Sas-4 is still localised in *dila* (E) and *unc* (F) mutants. (G) The uncoordinated phenotype of the weak hypomorph *dila*²⁴⁴ is enhanced after mutation of one copy of *unc*. The phenotype was scored according to three classes of increasing severity of uncoordination (see Materials and Methods). The difference in distributions is significant according to a χ -squared test, $P < 0.001$.

First, sensory cilia are truncated with disarrayed axonemes, which implies a shortage of building blocks, such as tubulin and associated proteins, being delivered into the cilium and is a common feature of IFT gene mutations. Second, HRP-reactive and EYS proteins remain at the ciliary base rather than being transported and secreted into the scolopale lumen. Third, axonemal microtubules mostly lack their dynein arms and other components required for their ordered arrangement. Studies in *Chlamydomonas* have shown that dynein arm precursors are preassembled and docked at the basal body for transport into the cilium through IFT (Hou et al., 2007). The phenotypes observed for *dila* all suggest impaired IFT in Ch neuron ciliogenesis. It is striking that *dila* mutant sperm have normally constructed axonemes, in contrast to sensory neurons. This difference correlates with the fact that IFT is not involved in sperm ciliogenesis (Han et al., 2003), thereby further implicating IFT insufficiency as the cause of the axoneme defects of sensory neurons. However, given the restricted localisation of DILA and its function in both neurons and sperm, it is unlikely that DILA forms part of the core IFT complexes. We propose that DILA ensures correct IFT function by facilitating the docking of IFT particles and vesicles (Marshall, 2008). This is emerging as a crucial regulatory function of the ciliary base. Notably, this function has been ascribed to the fibrous structures attached to both the basal body and the transition zone – both of which would be consistent with the DILA-FLAG localisation (Nigg and Raff, 2009; Silverman and Leroux, 2009). Consistent with this role, IFT components and cargoes have been found associated with these structures, presumably parked transiently (Deane et al., 2001; Stephan et al., 2007).

A few proteins are known to affect IFT by contributing directly to the structural integrity of the fibrous structures, including

Chlamydomonas CEP290 (Craig et al., 2010), at the transition zone, and CEP164 (Graser et al., 2007) and *Spermatozopsis* P210 (Lehtrekk et al., 1999) at the basal body. In *dila* mutant cilia, the structure of the basal body appears grossly normal, suggesting that *dila* plays a distinct role from these proteins, perhaps including a more direct role in docking rather than in structural integrity. The docking platform has been proposed not only to regulate selective entry into the cilium but also to be site of activation of anterograde kinesin motors, deactivation of retrograde dynein motors (Rosenbaum and Witman, 2002) and processing of tubulin (Stephan et al., 2007). We suggest that DILA participates directly in these processes, or that it might act as a scaffold to recruit the proteins that carry out these functions.

Genetic interaction and localisation evidence suggest that DILA and UNC proteins work together at the base of the cilium, perhaps defining an entity that is distinct from core structure of the basal body. Moreover, both are expressed before basal body conversion (Baker et al., 2004; Cachero et al., 2011). Interestingly, DILA and UNC are both coiled-coil proteins, which raises the possibility that they interact directly. Indeed co-dependence of localisation, such as we observed for these proteins, is a common feature of members of a protein complex, and in the context of ciliogenesis has been observed for IFT-A components and for TRPV channel subunits (Gong et al., 2004; Lee et al., 2008). *dila* and *unc* also show some phenotypic similarities with *yuri*, which also encodes a coiled-coil protein required for Ch neuron and sperm differentiation (Texada et al., 2008). The location of YURI protein in sensory neurons is not yet known.

The occurrence of *dila* phenotypes in both Ch neurons and sperm suggests a common function for DILA in these processes. One function for the basal body is to act as a template for axoneme

formation. However, the fact that axoneme construction appears to proceed normally in *dila* mutant sperm flagella appears to rule out a role for *dila* in axoneme templating. An important function of the basal body in sperm differentiation appears to be maintaining the association between the nucleus and the axoneme during elongation, and this seems to underlie the defects in *unc* and *yuri* mutants, possibly owing to a failure in centrosome to basal body conversion or in correctly orientating basal body positioning during individualisation (Baker et al., 2004; Texada et al., 2008). Although this phenotype remains to be explored fully, *dila* phenotypes, such as the disorganised sperm orientations within a cyst, are consistent with such a function. Ciliogenesis in sperm differentiation is highly unusual, and sperm differentiation itself is highly specialised. It might be that DILA and other proteins have been recruited to a different role required for a highly derived basal body function during spermiogenesis. Alternatively, similar to the neuronal phenotype, *dila* might be required to target or transport components required for sperm individualisation.

UNC and YURI have no clear orthologues in vertebrates, although it has been suggested that UNC is functionally equivalent to human OFD1, a centrosomal and basal body coiled-coil protein mutated in the ciliopathy, oro-facial-digital syndrome 1 (Baker et al., 2004; Romio et al., 2004). In contrast to UNC and YURI, DILA is predicted to be orthologous to human AZI1. AZI1 was first discovered in the sperm of mouse (Aoto et al., 1996) and is implicated to be present at the human centrosome (as CEP131) in a proteomic analysis (Andersen et al., 2003). In a recent study, morpholino knockdown of CEP131 in zebrafish resulted in developmental phenotypes consistent with defective cilia (Wilkinson et al., 2009). Conservation of protein structure, localisation and mutant phenotype imply that DILA, and its AZI1 homologues, is likely to be a fundamental component of the ciliary base. Therefore, our study suggests that *AZI1* is a candidate gene for human ciliopathies and that it might interact with *OFD1* during ciliogenesis.

Materials and Methods

Fly stocks

Flies are maintained on standard medium at 25°C. We used OreR or *w¹¹¹⁸* as wild-type controls. The P-element insertion line BG02674 and *unc²⁴* were obtained from the Bloomington Stock Center (Indiana University, Bloomington, IN). *unc-gfp* flies were described previously (Baker et al., 2004). The drivers used were *scaGal4* and *elavGal4* (Bloomington). PACT-GFP flies have been described previously (Martinez-Campos et al., 2004).

Imprecise excision

Imprecise excision mutagenesis of *dila* was performed using P element line P{GT1}CG30001^{BG02674} within *CG30001*, a gene downstream of *dila*. The P element was mobilised by crossing to a $\Delta 2-3$ transposase line. Male progeny from females containing both the P element and the transposase were chosen as candidate deletion alleles, balanced over CyO, Kr-GFP, then made homozygous and the presence of deletions determined by PCR using primers *dila* del A and *dila* del B (supplementary material Table S1). Approximately 300 excision lines were analyzed and three deletions were detected, named *dila⁸⁷*, *dila²⁴⁴* and *dila⁸¹*.

Fusion protein construction

Primers used to clone the full length *CG1625-RA* cDNA are listed in supplementary material Table S1 (*CG1625* cDNA 5' and 3'). RNA was extracted from wild-type adult flies using the RNeasy Plus Mini kit (Qiagen). First-strand synthesis was performed using the ImProm-II reverse transcription system (Promega). The PCR product was cloned into pSC-A. To construct the pUAS-FLAG-*dila* fusion, three repeats of FLAG (DYKDDDDK) were introduced into the N- or C-terminus of the *CG1625-RA* cDNA in pSC-A using site-directed mutagenesis (QuickChange IXL, Stratagene). After sequencing, the modified FLAG-DILA fusion fragments were cloned into pUAST digested with *EcoRI* and *XhoI*. The resulting construct was transformed into *Drosophila* embryos by microinjection. Both N- and C-terminal fusions rescue the *dila⁸¹* behavioural phenotype. Experiments shown in Fig. 5 employ the C-terminal fusion.

Sample preparation

Embryos at the desired stage were collected on grape agar plates, dechorionated and fixed for 15 minutes with 3.7% formaldehyde in PBS. Adult testes were dissected in PBS. Samples subjected for RNA in situ hybridisation were fixed in 3.7% formaldehyde in PBS for 20 minutes, and stored in methanol at -20°C overnight before use. For antibody staining, testes were squashed and prepared as previously described (Blachon et al., 2008). For staining of larval chordotonal organs, third-instar larvae were dissected in a drop of PBS. The larval pelt was fixed with 3.7% formaldehyde in PBS for 20 minutes.

RNA in situ hybridisation

dila probes were synthesised by PCR amplification with *dila* 5' and *dila* T7 primer sets (supplementary material Table S1), using cDNA extracted from wild-type embryos as template. The probe was digoxigenin labelled (Roche) and purified with RNeasy Mini Spin Columns kit (Qiagen). In situ hybridisation was carried out on the embryos and testes by standard protocols. For embryos, homozygote *dila* mutant embryos were distinguished from heterozygote siblings by the absence of Kr-GFP expression from a transgene carried on the balancer (CyO, Kr-GFP).

Immunohistochemistry

Primary antibodies used in these studies were as follows: mouse MAb22C10 (1:200), and mouse anti-EYS antibody (1:200) (both from Developmental Biology Hybridoma Bank, Iowa), mouse and rabbit anti-GFP antibody (1:500, Molecular probes, Invitrogen) and mouse anti-FLAG antibody (1:500, Sigma, F1804). The following centrosome antibodies were also used: rabbit Sas-4 (1:250) (Martinez-Campos et al., 2004), rabbit CP190 (1:500) (Kellogg and Albert, 1992) and mouse γ -tubulin (1:500, Sigma, GTU88). Secondary antibodies (1:500) were from Molecular Probes (Invitrogen). Samples were mounted with vectashield (Vector laboratories). Confocal microscopy was carried out using Zeiss PASCAL and Leica TCS SP2 microscopes.

Transmission electron microscopy

Transmission electron microscopy analysis of testis and Johnston's Organ from the adult antenna were performed as previously described (Lee et al., 2008). Sample embedding, sectioning and electron microscope examination were performed by Electron Microscopy Research Services of Newcastle University Medical School using a Philips CM100 Compustage (FEI) microscope with images collected using an AMT CCD camera (Deben).

Phenotypic tests

For the bang test, 2- to 4-day-old flies were collected under CO₂ after eclosion. Assays were carried out in a 100 ml cylinder. After a few minutes of acclimatisation, the cylinder was manually banged twice on a padded surface. Flies were left for 5 minutes before counting the number of flies above the 50 ml mark. Tests were repeated three times for each strain and flies were rested for 5 minutes between tests. For male fertility tests, individual males (2-day-old) were placed with two virgin OreR females in vials for 7 days at 25°C. The parent flies were then removed and all resulting F1 progeny was counted. Seven control crosses and 14 *dila⁸¹* mutant crosses were analysed. To test the genetic interaction between *dila* and *unc*, the uncoordinated phenotype of adult flies was scored according to the following: class I flies are able to stand and walk properly; class II flies walk in an uncoordinated way, falling easily and with wings held erect; class III flies are completely unable to walk and become stuck in the food.

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Supplementary material available online at

<http://jcs.biologists.org/cgi/content/full/124/15/2622/DC1>

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